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The purpose of this project is to identify genes responsible for inherited susceptibility to breast cancer in families. Two such genes have been cloned (BRCA1 and BRCA2), but the existence of families with many cases of breast cancer but no mutations in these genes suggests that other BRCA genes may exist. Using an integrated approach involving dissection of germline chromosomal rearrangements in women with very early onset breast cancer, coupled with linkage analysis in families, we are cloning the gene on chromosome 10q responsible for Cowden disease and possibly for breast cancer in the absence of other Cowden symptoms.

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Mary Claire King — Sept 10, 1996

PI - Signature — Date

GENETIC ALTERATIONS IN FAMILIAL BREAST CANCER: MAPPING AND CLONING GENES OTHER THAN BRCA1

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GENETIC ALTERATIONS IN FAMILIAL BREAST CANCER: MAPPING AND CLONING GENES OTHER THAN BRCA1

INTRODUCTION

Two genes responsible for inherited breast cancer--BRCA1 and BRCA2--have been cloned. However, in our series of families with multiple cases of breast cancer, several have no identified BRCA1 or BRCA2 mutations (after screening the entirety of both genes) and no apparent linkage of breast cancer to either 17q21 or 13q12 (see our 1995 progress report). The goal of our project is to identify genes responsible for inherited breast cancer in these and other "unexplained" families. In the course of carrying out a genome-wide screen for linkage in these families, it became clear that the frequent combination of late age at breast cancer onset and small family size would limit the statistical power of linkage analysis. Therefore, we decided to integrate linkage analysis with the analysis of germline chromosomal abnormalities that might predispose to breast cancer.

In order to identify germline chromosomal abnormalities that might alter breast cancer genes, we sought patients who had both very early onset breast cancer and developmental abnormalities. It is our hypothesis that this combination of phenotypes will often reflect de novo, germline chromosomal rearrangements in genes critical to breast cancer, as well as of genes critical to normal development. The most promising case discovered so far in this search is that of a young woman (LP) with early bilateral breast cancer in the context of Cowden's syndrome and a complex de novo rearrangement of chromosomes 10q, 2q, and 13q (see Body of this report).

Although Cowden disease is rare, it is our hypothesis that the Cowden disease gene might influence breast cancer risk in families other than those with Cowden disease. It is well-documented that some mutations in a gene may lead to a very severe phenotype and other mutations in the same gene to a more restricted phenotype. The high frequency of breast cancer in female Cowden patients (~30%) makes it a strong candidate for a breast cancer susceptibility gene (Hanssen and Fryns 1995).

Cowden's disease is a multiple hamartoma syndrome with an autosomal dominant pattern of inheritance (Weary et al. 1972), originally described in the family of Rachel Cowden (Lloyd and Dennis 1963). Cowden disease is characterized by multiple nodules of the skin and mucous membranes, fibromas of breast and thyroid, and gastrointestinal polyps (Brownstein et al. 1977). Mental retardation, macrocephaly, seizures, and ataxia are frequently involved. Although Cowden syndrome has traditionally been defined by skin lesions, it also involves cancer of internal organs, most frequently thyroid and breast.

Breast cancer frequently occurs in the context of Cowden's disease (Walton et al. 1986). In seven families with 21 cases of Cowden disease, inheritance was autosomal dominant with high penetrance and high frequency of breast cancer in females, craniomegaly, gastrointestinal polyps, and fibromas (Starink et al. 1986). In another Cowden disease family, proband had multiple pappillomas and was

diagnosed at age 32 with breast cancer. Her mother died of breast cancer at age 42, and 2 maternal aunts had premenopausal breast cancer (Williard et al. 1992). In still another large Cowden disease family, there was greater disease severity and earlier onset in successive generations (Hanssen et al. 1993).

The gene for Cowden disease has recently been mapped to chromosome 10q22-q23 by linkage analysis in 12 families, with a maximum lod score of 8.92 at theta = 0.02 with the marker D10S573 (Nelen et al. 1996). The neurologic and neoplastic features of Cowden disease in these families are consistent with the possibility that the Cowden gene is a tumor suppressor gene.

BODY OF REPORT

Phenotype and karyotype of patient with informative chromosomal rearrangement. Patient LP was identified by our colleague Dr. Fernando Arena, Department of Pediatrics, University of Miami, who has collaborated with us to search for patients with early onset breast cancer and mental retardation. LP had intraductal adenocarcinoma of the left breast at age 24 and of the right breast at age 32. She also has macrocephaly, mental retardation, enlarged thyroid nodule, parotid and muscular hemangiomas. Dr. Arena and Dr. Herbert Lubs diagnosed her phenotype as Cowden disease. Karyotypic analysis revealed a complex chromosomal rearrangement 46,XX,t(2;10;13)(q32.2;q23.2;q13.2). A chromosomal segment from 2q moved to 10q; material from 10q moved to 13q; part of 13q moved to 2q (Figure 1). Her parents are karyotypically normal, indicating that her chromosomal rearrangement is de novo. There is no family history of breast cancer or of other symptoms of Cowden disease.

Mapping the breakpoint on chromosome der13. At the time LP was identified, BRCA2 had been mapped to 13q12-q14, but not yet cloned. Since the region of BRCA2 linkage was apparently close to the 13q breakpoint, we first mapped the 13q/10q breakpoint on der13. YACs were selected using markers from 13q12-q14 as STSes (Figure 2). The YACs were mapped by fluorescence in situ hybridization (FISH) to chromosomes of LP, with results indicated in Figure 2. YACs 847 and 800, which had been selected with D13S263, spanned the der13 breakpoint. (In addition to providing physical reagents spanning the der13 breakpoint, these FISH data indicated that the breakpoint was distal to BRCA2, which was subsequently confirmed when BRCA2 was cloned. We were pleased that BRCA2 and the LP 13q breakpoint were different, because this means that another breast cancer-related gene can be discovered by this project.)

YACs 847 and 800 were subcloned into cosmids and ~1000 cosmids gridded into arrays. Twenty cosmids were selected at random and mapped by FISH on LP chromosomes. Two were on der13 and hence proximal to the 13q breakpoint; the others were on der2 and hence distal to the 13q breakpoint. D13S263 mapped to one of the proximal cosmids.

In February 1996, BAC libraries became commercially available, and our lab was the first to take advantage of them. D13S263 was used to screen the library, identifying four BACs. In addition, an end sequence from cosmid 7.1.G4 was used to screen the BAC library, identifying three more BACs. These seven BACs were mapped to LP's chromosomes by FISH: one BAC derived from 7.1.G4 spans the 13q break. As of May 1996, therefore, we had a BAC of 125 kb spanning the chromosome 13q breakpoint.

Mapping the breakpoint on chromosome 10q. In May 1996, Cowden disease was mapped to 10q22-q23, with its locale predicted by linkage data to be D10S573--D10S215--CD--D10S564--D10S583 (Nelen et al. 1996). To test whether the LP chromosome 10q breakpoint was within the Cowden linkage region, and hence whether her karyotype was consistent with the linkage data, markers D10D573 and D10S583 were used to screen the BAC library. One BAC was identified with each marker and mapped onto LP chromosomes by FISH. BAC 573 was on der10 and hence proximal to the 10q breakpoint; BAC 583 was on der13 and hence distal to the breakpoint. Next,

Figure 1. Complex de novo chromosomal rearrangement of breast cancer patient LP

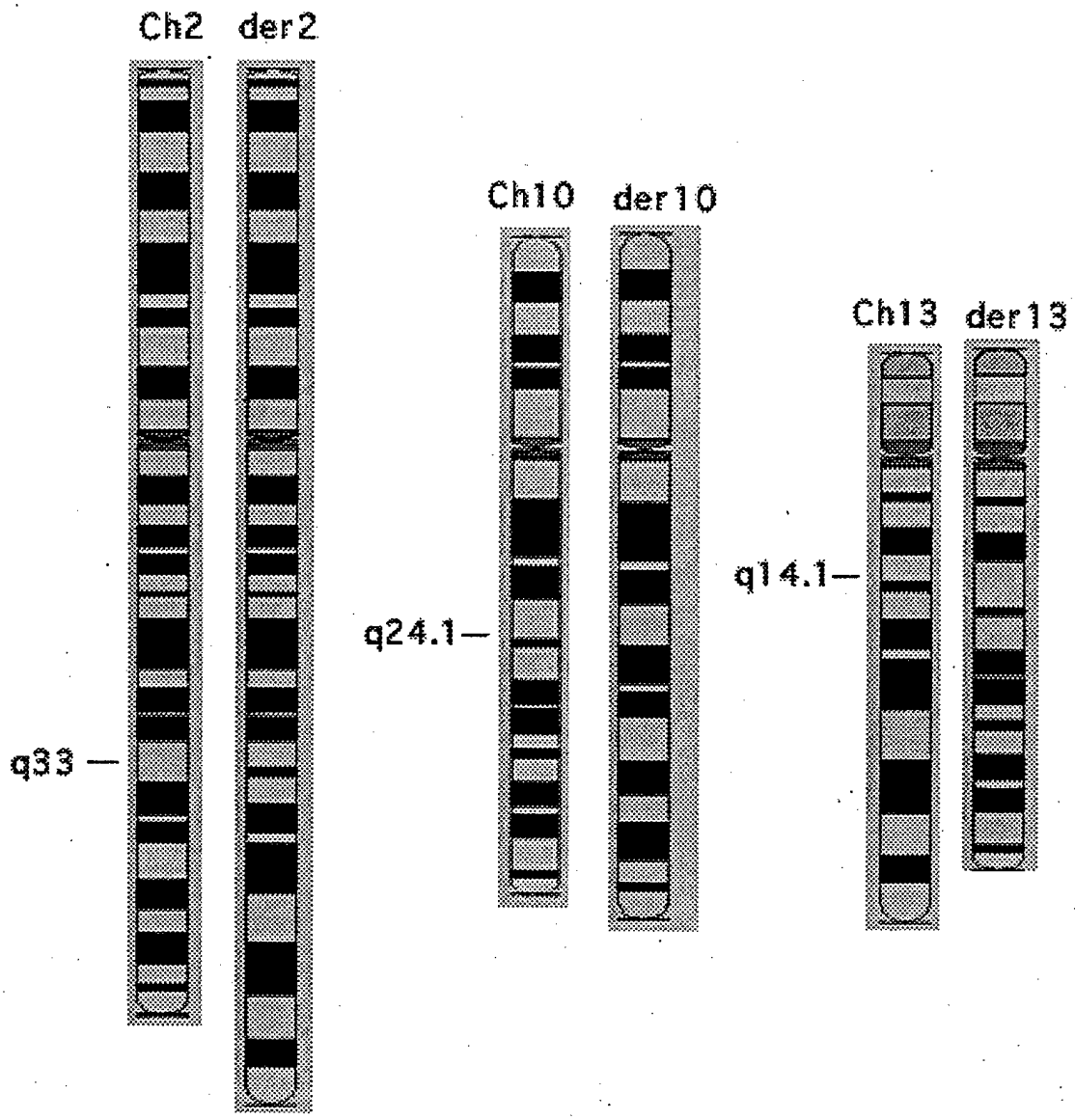
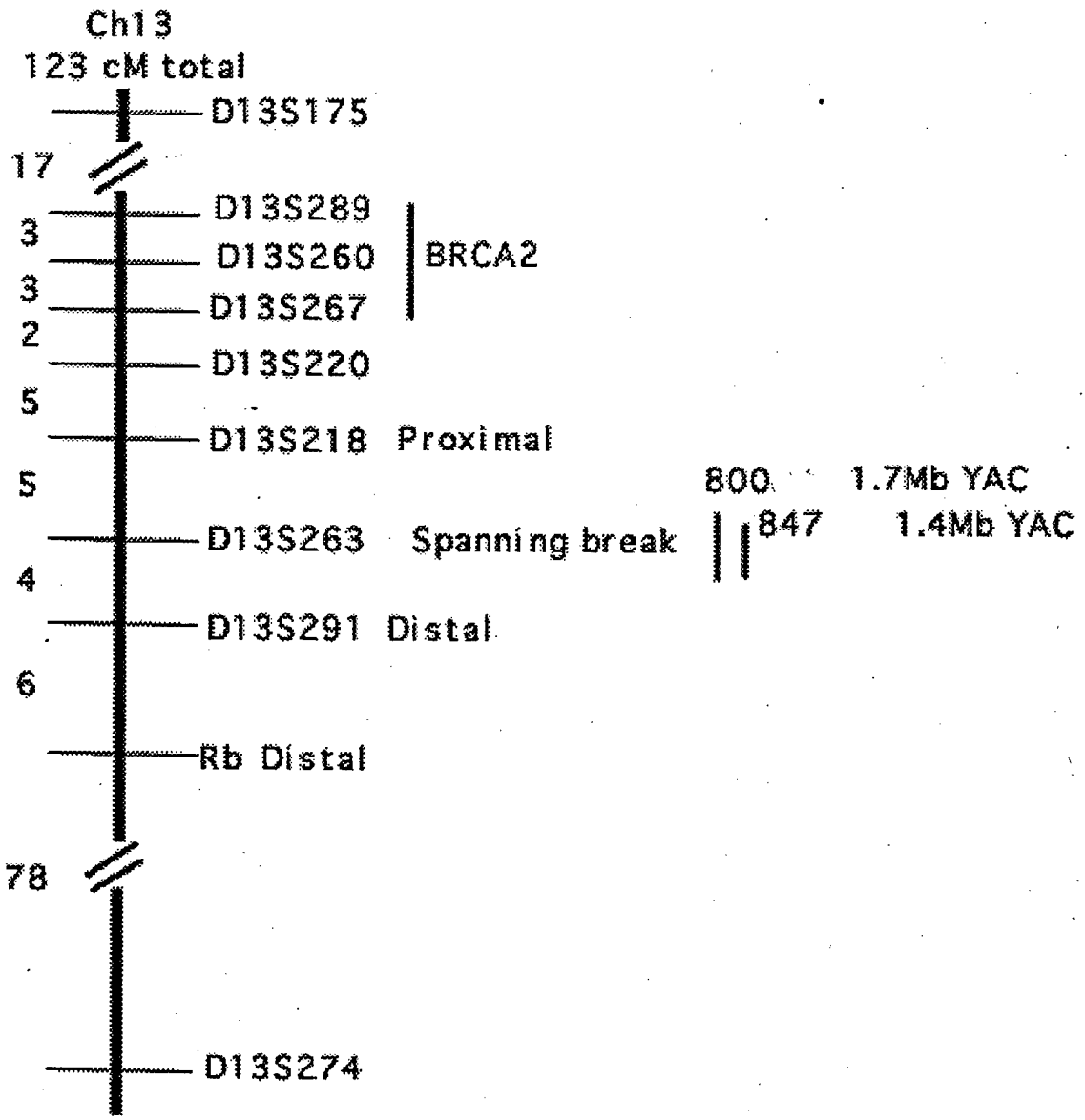


Figure 2. Location of chromosome 13 breakpoint of breast cancer patient LP



markers D10S215 and D10S564, reportedly flanking Cowden disease at 5 cM, were used to screen the BAC library. Two BACs were identified with each marker and mapped onto LP chromosomes by FISH. The 215 BACs were on der10 and hence proximal to the breakpoint; the 564 BACs were on der13 and hence distal to the break. Therefore LP's chromosomal rearrangement is consistent with her phenotype being caused by an alteration in the Cowden gene localized to 10q22-q23.

Next, the YAC contig of the D10S215-D10S564 region was accessed from the Web site of the Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu) (Figure 3). Sizes of the YACs were accessed from the Genethon web site (www.genethon.fr/genethon_en.html). Based on the sizes of the non-chimeric YACs, we estimate the size of the D10S215-D10S564 region as ~ 5 Mb. STSes between D10S215 and D10S564 on the contig were used to design primers which were then used to screen the BAC library. Six BACs were identified and confirmed by mapping back the markers to their host BACs. No BAC contained more than one marker. All BACs were mapped to LP chromosomes by FISH. Locales permitted the region of the 10q breakpoint to be further constrained.

Future mapping and cloning steps:

(1) The next step will be to sequence ends of proximal and distal BACs and walk between them, mapping BACs to LP chromosomes at each step by FISH. A BAC spanning the chromosome 10q breakpoint will be obtained.

(2) The BACs spanning the chromosome 10 and 13 breakpoints will be digested with an enzyme that cuts at 10-20 kb intervals and individual fragments mapped by FISH. Fragments spanning each breakpoint will be selected.

(3) A lambda genomic library will be prepared from LP DNA. Subclones spanning the breakpoints from the chromosome 10 BAC and the chromosome 13 BAC will be used to screen the genomic library. Hybridizing lambda clones and the BAC subclones will be digested with several frequent cutters and fingerprinted by running LP lambda clones and BAC controls side-by-side. Differences between LP clones and the BAC subclones may represent rearranged fragments from LP.

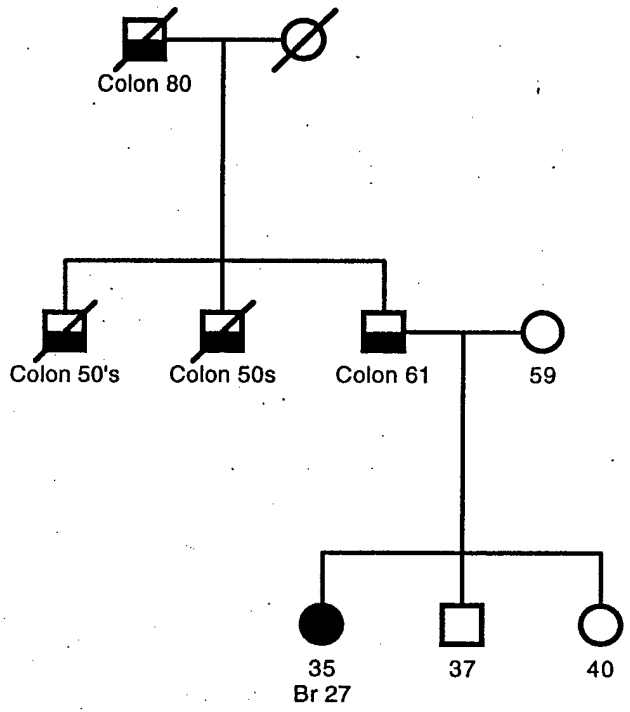
(4) Potential rearranged fragments from LP will be sequenced and the sequences searched for open reading frames and for matches to fragments of known genes and ESTs. Also, the fragments will be used to screen appropriate cDNA libraries for genes that may be part of the rearranged complex.

Other patients with breast cancer and inherited Cowden disease. Our series of breast cancer kindreds includes family 97 (unrelated to LP, of course), whose proband had breast cancer at age 27 and has skin hamartomas consistent with Cowden disease. Her father, two paternal uncles, and paternal grandfather had Cowden disease and colon cancer (Figure 4). Her siblings are unaffected with either cancer or the skin lesions of Cowden disease. The affected members of family 97 will offer a confirmation of a candidate gene identified in LP.

YAC Contig of Chromosome 10q between D10S215 and D10S564: Whitehead-MIT Genome Center

WC10.7	kb	D10S215	AFMa086WG9	D10S541	AFM280WE1	WI-10275	WI-8733	WI-6971=APT1	D10S1739	WI-6075	WI-9217=MPP1	WI-4264	AFMA048WB9	AFM225YD12	CHLC.GATA66C04	AFMA114XB1	D10S1753	D10S564	Key
855_G_4	1190	F																	F=fingerprint
738_B_12	1300	D																	D=definite
787_D_7	chi	D	D																S=STS
746_H_8	1200	C	S	S															C=CEPH
968_E_6	chi			D															chi=chimeric
773_C_2	780	F	S																
921_F_8	1570	C		C															
922_E_6	chi	C					F												
894_H_5	chi	D																	
876_G_11	chi	F																	
821_D_2	1150	F		F															
934_D_3	chi	S	S	D	D														
799_E_5	1780		F																
796_D_5	800		S	F															
964_A_8	chi			D															
927_H_12	890			D	D		D	D											
757_D_8	chi			D															
829_E_1	chi			C															
924_F_11	1410			F															
831_D_9	1120			C															
734_B_4	280			F															
855_D_2	220			S	S														
788_F_4	1400				F		F	F	D	F									
947_H_9	chi				D	D	D	D	S										
786_C_8	810				F	F													
794_H_1	chi						D	D	D	D									
741_H_7	830						D	D	D	F									
758_H_7	chi						D	D	D	F	S								
724_A_3	chi									F									
944_C_1	1370									D	D	F	F						
750_H_1	?									D	F	F	S	D	F				
906_D_1	1010										D				F	D	D		
847_D_4	1430										D	D		D	F	D	F		
931_D_3	1240										D			F		F	D		
800_A_11	chi										D	S	S	D	D	D	F	S	
750_G_12	chi										F								
759_C_9	chi										F	F	S	F		F	F		
759_D_11	chi											F	S	F	F	F	F		
931_D_2	chi											D							
847_C_4	280												S				S		
967_G_G	chi												D	D	S	D	D	S	
709_F_11	560												D	F	F				
950_A_1	870												F	D		D	F	S	
931_H_3	chi												D						
854_H_12	920												F	D	D		F	S	
890_G_6	635												F		F	F	F	S	
962_G_11	1610													D		D	D	S	
710_F_1	1260													F	F				
773_F_6	1700													F	F	F	F		
718_D_7	chi														D	F	D		
720_G_4	chi														F				
752_H_1	chi														F				
773_F_5	chi														S	S	S	S	
885_H_11	chi																F	C	
748_D_8	730																	F	
647_G_1	360																	C	
660_A_8	140																	c	
653_F_6	chi																	C	

Figure 4. Family 97 with Cowden disease and breast cancer



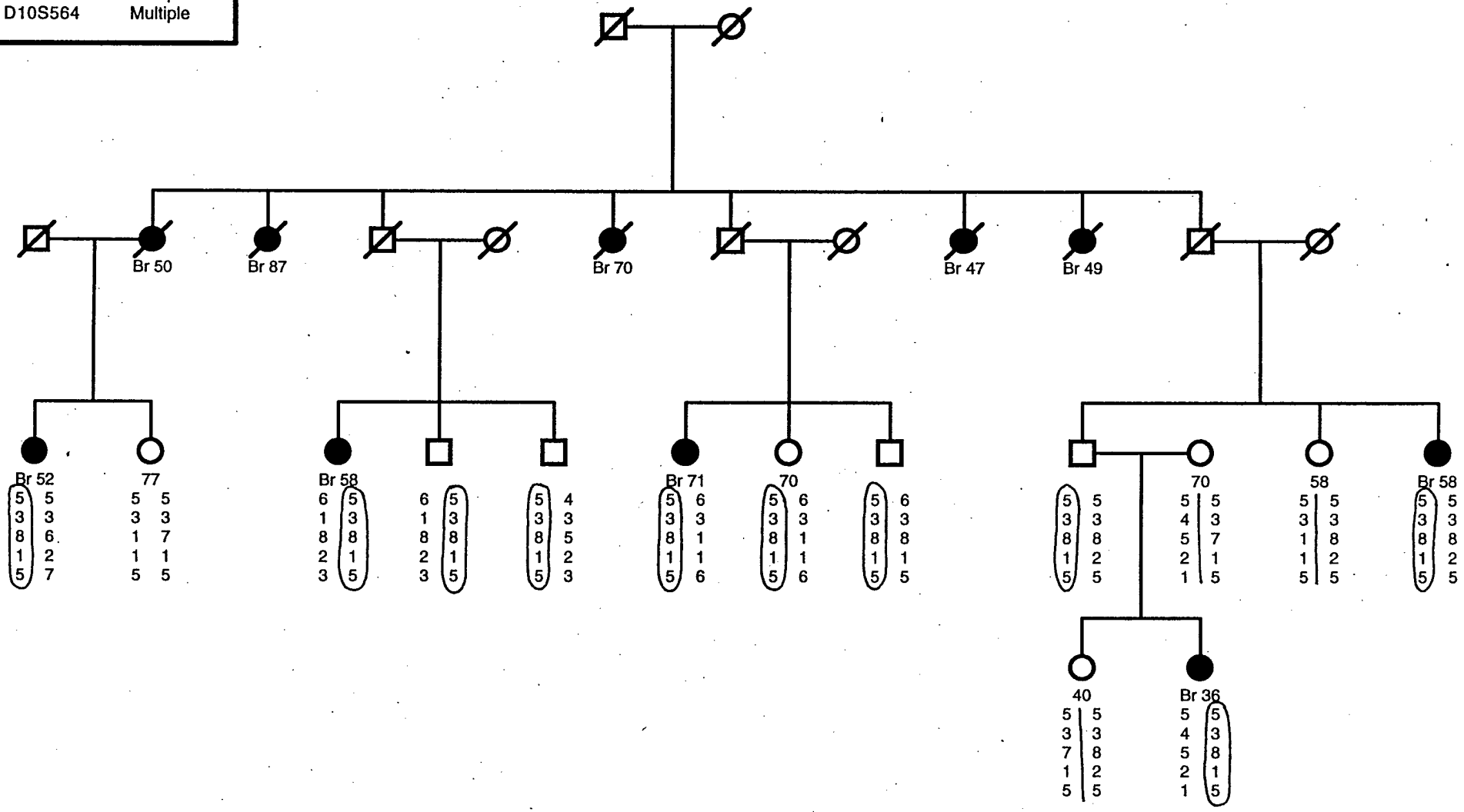
Ten additional, unrelated breast cancer patients with Cowden disease have been identified through a collaboration with Dr. Monica Peacock of Columbia University. Ages at breast cancer diagnosis in these patients range from 33 to 74. All ten patients had ductal carcinoma in situ (DCIS), with additional infiltrating breast cancer in nine patients. They and multiple members of their families have other symptoms of Cowden disease. Cell lines on these patients have been established and will be screened for mutations in candidate genes identified from LP.

Linkage analysis of chromosome 10q markers to breast cancer in high-risk families without Cowden disease. Polymorphic markers flanking the Cowden disease gene on 10q were genotyped in six families from our series with 5 or more cases of breast cancer, but no BRCA1 or BRCA2 mutations and no evidence of linkage of breast cancer to 17q21 or 13q12 (Figures 5A-F). No other symptoms of Cowden disease were present in any family. Markers were selected from the published report of Cowden disease linkage (D10S215 and D10S564) and from the YAC contig WC10.7 (AFM086wg9, D10S541, and AFM280we1). Haplotypes were constructed wherever possible; genotypes of deceased persons were reconstructed if this was possible without ambiguity.

Given the late age at onset of breast cancer and the large number of deceased individuals in these families, it is difficult to tell from linkage data alone whether the Cowden gene may be responsible for breast cancer in any family. For all except Family 20, sporadic cases of breast cancer would have to be postulated. An informative, direct test of the relevance of any Cowden disease gene to breast cancer in these families will be to screen DNA and RNA from affected relatives for mutations in candidate genes identified from LP.

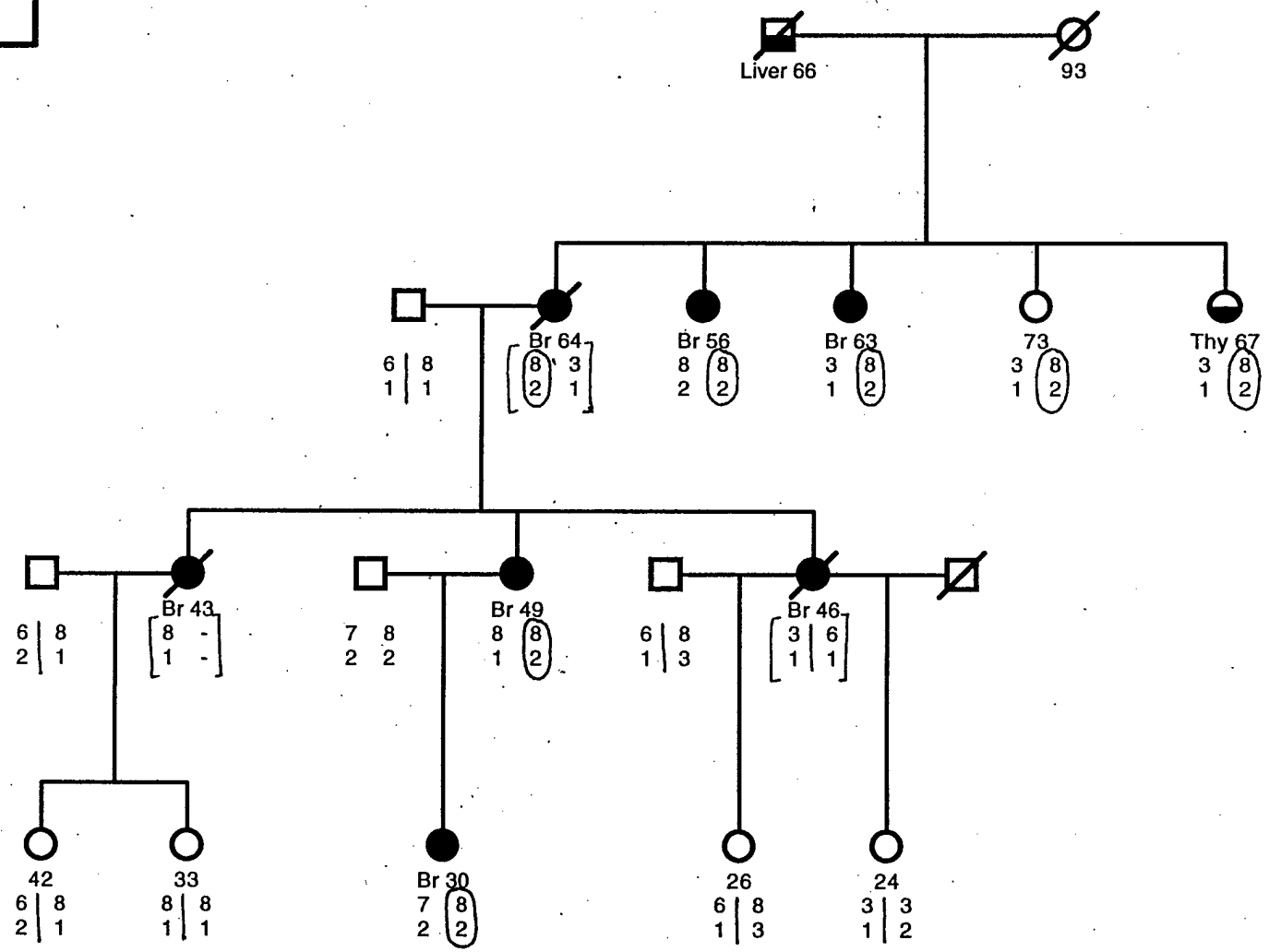
Markers	Alleles
D10S215	Multiple
AFM086wg9	Multiple
D10S541	Multiple
AFM280we1	Multiple
D10S564	Multiple

Figure 5A. Linkage of breast cancer to markers on chromosome 10q



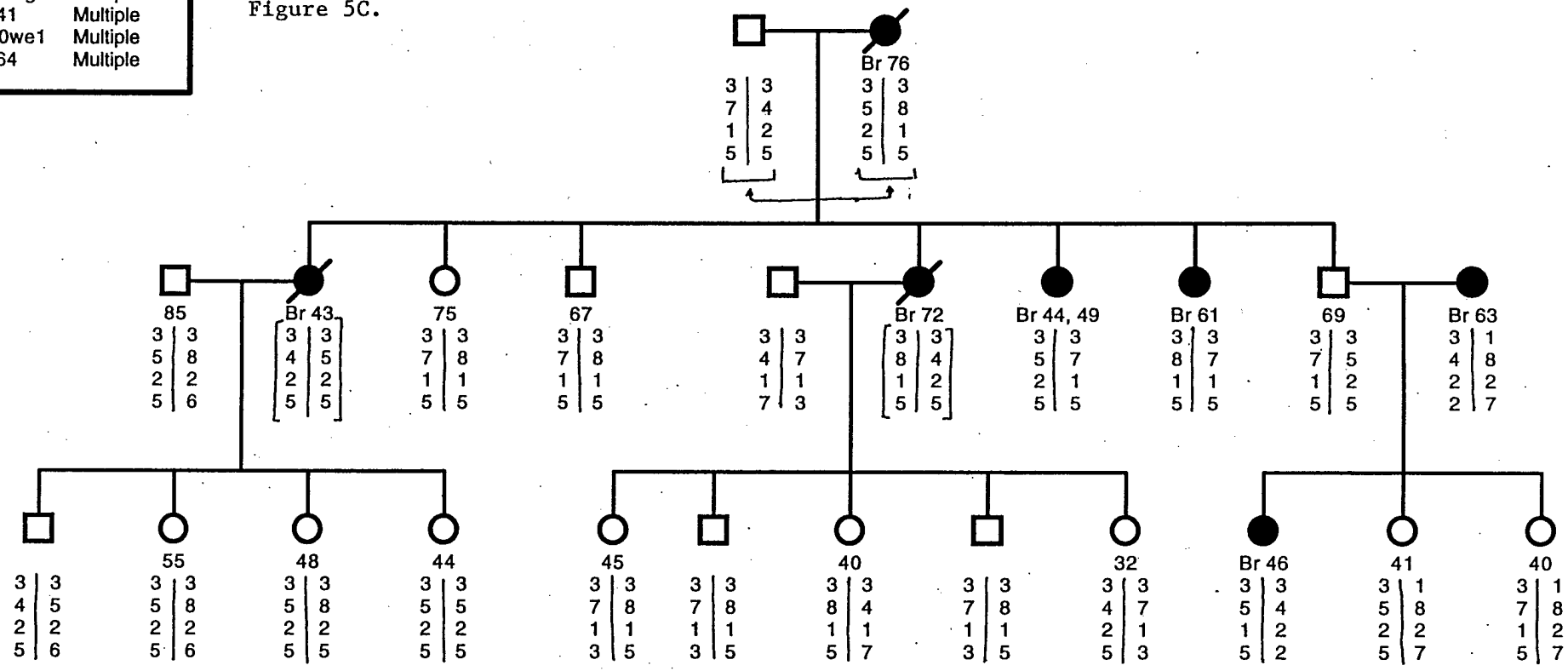
Markers	Alleles
D10S541	Multiple
AFM280we1	Multiple

Figure 5B.



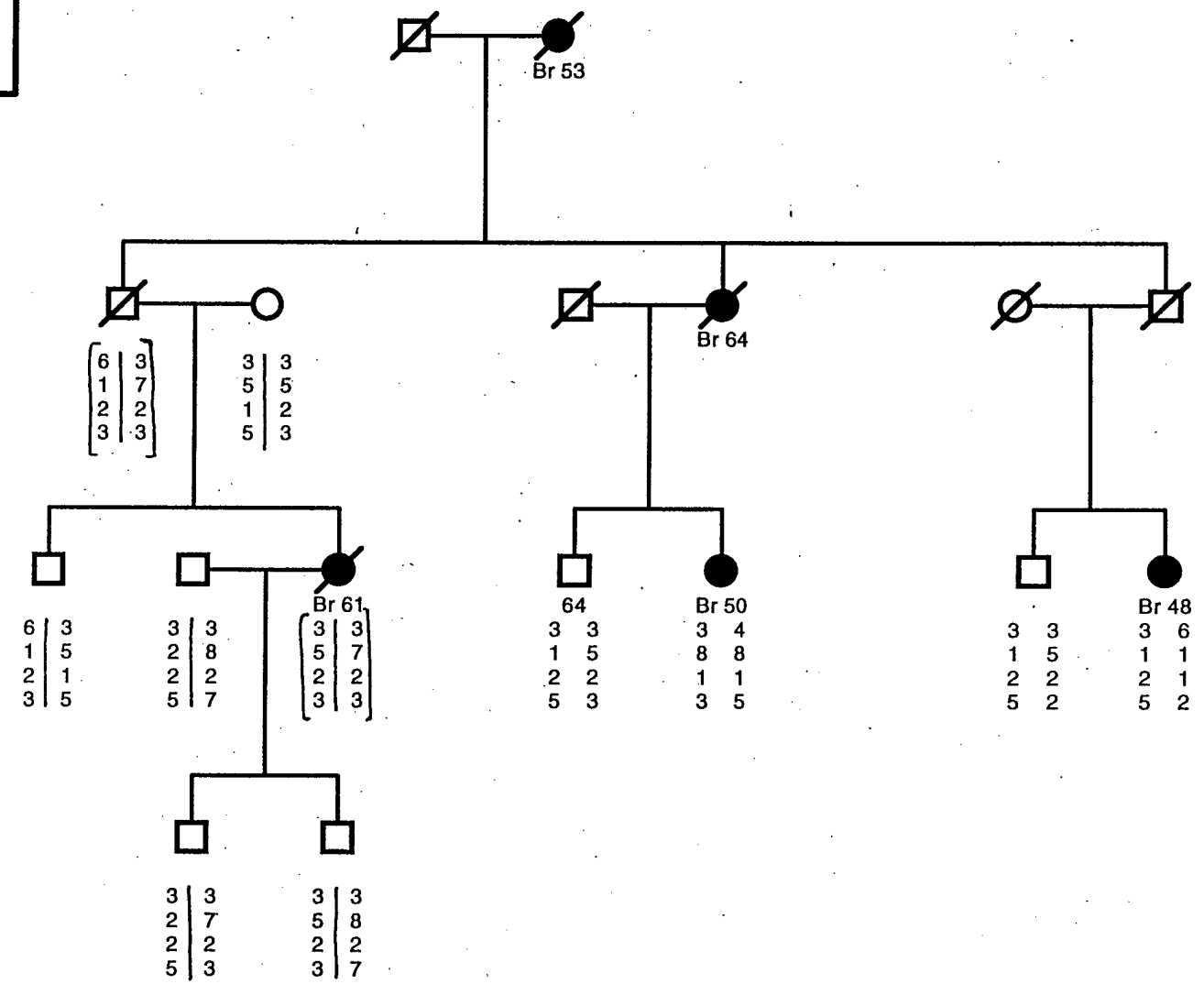
Markers	Alleles
AF[illegible]086wg9	Multiple
D1[illegible]3541	Multiple
AF[illegible]280we1	Multiple
D1[illegible]3564	Multiple

Figure 5C.



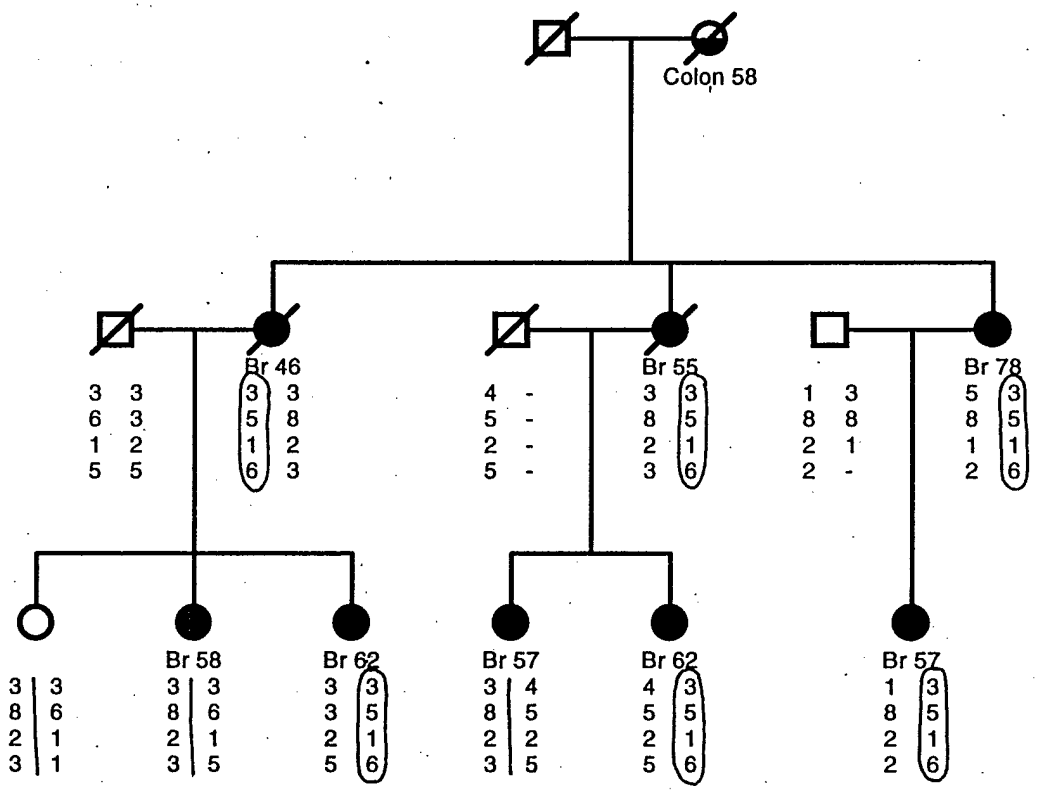
Markers	Alleles
AFM086wg9	Multiple
D10S541	Multiple
AFM280we1	Multiple
D10S564	Multiple

Figure 5D.



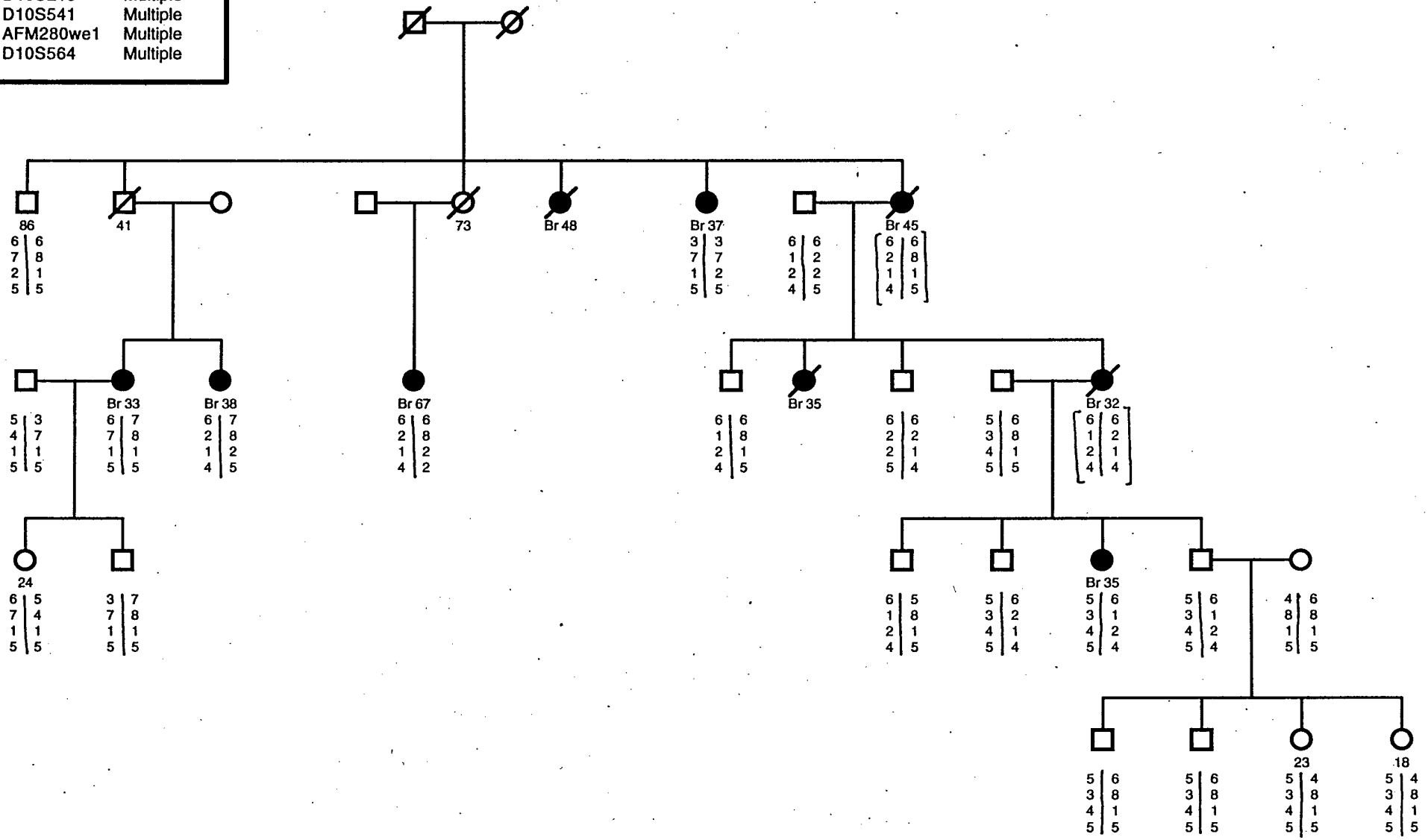
Markers	Alleles
AFM086wg9	Multiple
D10S541	Multiple
AFM280we1	Multiple
D10S564	Multiple

Figure 5E.



Markers	Alleles
D10S215	Multiple
D10S541	Multiple
AFM280we1	Multiple
D10S564	Multiple

Figure 5F.



CONCLUSIONS

(1) Patients with breast cancer and germline chromosomal alterations provide an excellent tool for identifying additional genes for inherited breast cancer.

(2) Patient LP, with young bilateral breast cancer and Cowden disease, has a complex germline chromosomal translocation involving 10q, 13q, and 2q.

(3) The 10q breakpoint of LP is in the region of linkage for the Cowden disease gene.

(4) BACs spanning the 13q/10q breakpoint have been identified. BACs spanning the 10q/2q breakpoint will be identified soon.

(5) Additional, unrelated patients with breast cancer and Cowden disease have been identified to test candidate genes for mutations.

(6) In families at high-risk of breast cancer without Cowden disease, linkage analysis of chromosome 10q markers to breast cancer indicate which affected relatives may be most informative for testing the hypothesis that a Cowden disease gene influences breast cancer susceptibility in other families.

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